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<p><b>(54) Title:</b> MICROFABRICATED APPARATUS FOR CELL BASED ASSAYS</p>			
<p><b>(57) Abstract</b></p> <p>Apparatus and methods are provided for performing cell growth and cell based assays in a liquid medium. The apparatus comprises a base plate supporting a plurality of micro-channel elements, each micro-channel element comprising a cell growth chamber, an inlet channel for supplying liquid sample thereto and an outlet channel for removal of liquid sample therefrom, a cover plate positioned over the base plate to define the chambers and connecting channels, the cover plate being supplied with holes to provide access to the channels. Means are incorporated in the cell growth chambers, for cell attachment and cell growth. In particular, the invention provides a rotatable disc microfabricated for performing cell growth and cell based assays. The apparatus and method can be used for the growth of cells and the detection and measurement of a variety of biochemical processes and products using non-invasive techniques, that is techniques which do not compromise the integrity or viability of cells.</p>			

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Microfabricated Apparatus for Cell Based Assays

The present invention relates to cell based assays. In particular the invention relates to a microfabricated apparatus for performing cell growth and cell based assays and to methods for performing such assays.

The current focus in high throughput screening applications towards the screening of increasing numbers of compounds is being driven by the twin technologies of combinatorial chemistry and genomics, in order to produce new potential drug targets and novel candidate drugs as potential therapeutic compounds. The primary screening process has been addressed by the development of high throughput screening assay processes and assay miniaturisation utilising the microtitre well plate format with 384, 864, 1536 or greater miniaturised wells. Miniaturised assays are capable of allowing throughput levels of over 100,000 tests/day in primary screening. At this level of throughput, a primary screen might be expected to yield 100-1000 'hits' per day. Each of these putative drugs is required to undergo further refined screening and testing in a variety of assays in order to investigate the biological compatibility of the compound. Such assays include bioavailability, metabolism and toxicology, and they are carried out predominantly using cultured cell lines. In comparison with assays used in the primary screening process, secondary screening assays have a much higher level of complexity and more stringent requirements, both in the mechanics of the assay and in the information generated. There is a requirement in the art for secondary screening assay methodologies which are capable of handling both the increasing rate of putative drug lead generation and the generation of biological data concerning the drug candidate. In addition, development of assays yielding higher information content have the potential to increase the level of characterisation of a lead drug during the screening phase of drug development.

Microfabricated devices have been described previously which are suitable for use in miniaturized biological analyses. For example, WO 96/15450 discloses a device which comprises an etched glass structure with a collection of chambers connected by a micro-channel and enclosed by a glass cover plate. Devices have been described by Wilding et al., for example in WO 93/22058, which discloses a mesoscale device for amplifying DNA by PCR, the device consisting of a number of chambers connected by a channel. WO 93/22055 and WO 93/22053 relate to devices for analysing a fluid cell-containing sample comprising a mesoscale flow system with entry port for capture and/or lysis of cells in biological fluids, or containing a binding moiety for specifically binding an analyte, where the analyte may be an intracellular component in a cell in a sample, or a cell population in a sample. The above devices are concerned with the measurement or detection of cells or cellular analytes in cells introduced into the device immediately prior to analysis. They are not described for use in cell culture or growth, nor for use in the study of cellular responses to agents under test. Moreover, they do not describe or permit the use of adherent cultured cells.

There is a requirement for a device capable of providing an environment which supports the long term survival of cultured cells, coupled with means to utilise cells cultured within the device for secondary drug screening or other studies.

In one aspect, the present invention provides apparatus microfabricated for performing cell growth and cell based assays in a liquid medium, said apparatus comprising:

- a base plate supporting a plurality of micro-channel elements, each comprising a cell growth chamber, an inlet channel for supplying liquid sample thereto and an outlet channel for removal of liquid sample therefrom;

- b) a cover plate positioned over said base plate said cover plate extending over said elements so as to define said chambers and connecting channels; said cover plate being supplied with holes to provide access to said channels; and
- 5 c) means, incorporated in said cell growth chambers, for cell attachment and cell growth.

In a second aspect of the invention there is provided a method for studying the effect of a test substance on a cellular activity or physical parameter by the use of the apparatus as defined, which method comprises:

- 10 a) providing a suspension of cells in a fluid medium;
- b) introducing said cells into said apparatus and causing said cells to be transported to one or more cell growth chambers in said apparatus;
- c) providing one or more samples of test substances whose effect upon
- 15 the cells is to be measured under conditions so as to cause said cells to be exposed to said substances;
- d) determining the effect of the test substances on said cells by means of optical detection.

20 Preferably the method for studying the effect of a test substance includes the step of culturing cells adhering to a surface within the apparatus prior to the introduction of the test substances. Preferably there are provided following step c) one or more assay reagents and dispersing the reagents to one or more reaction chambers in the apparatus.

25 In a further aspect of the present invention there is provided a method for measuring a cellular analyte by the use of the apparatus as defined, which method comprises:

- a) providing a suspension of cells containing an analyte to be measured
- 30 in a fluid medium;

- b) introducing cells into the apparatus and causing the cells to be transported to one or more cell growth chambers in the apparatus;
- c) allowing cells to grow;
- d) providing one or more assay reagents and dispersing the reagents to 5 one or more chambers in the apparatus;
- e) measuring the cellular analyte by optical means.

In order that the invention may be better understood, several embodiments will now be described by way of example only and with 10 reference to the accompanying drawings in which:

Figure 1a is a diagrammatic representation in plan of an individual micro-channel element of the microfabricated apparatus for performing cell growth and cell based assays;

15 Figure 1b is a sectional view of a microfabricated disc including a plurality of assay elements for performing cell growth and cell based assays according to the present invention;

Figure 2 represents an alternative configuration of an individual assay element of the microfabricated apparatus for use with cells growing on the 20 surface of microcarrier beads; and

Figure 3 represents a further configuration of an individual assay element of the microfabricated apparatus in which means are provided for preventing or impeding the passage of cells in the cell growth chamber.

25 Referring to Figure 1b, the apparatus of the present invention comprises a rotatable disc (18) microfabricated to provide a sample introduction port (not shown) located towards the centre of the disc and connected to an annular sample reservoir (9) which in turn is connected to a plurality of radially dispersed micro-channel assay elements (6) each of 30 said micro-channel elements comprising a cell growth chamber, a sample inlet channel and an outlet channel for removal of liquid therefrom and a

cover plate positioned onto said disc so as to define closed chambers and connecting channels. Each micro-channel element is connected at one end to the central sample reservoir (9) and at the opposing end to a common waste channel (10).

5

Each of the radially-dispersed micro-channel elements (6) of the microfabricated apparatus (shown in Figure 1a) comprises a sample inlet channel (1) connected at its left hand-end end to the reservoir (9), a cell growth chamber (2) for performing cell growth and connected through a 10 channel (4) to an assay chamber (3) and an outlet channel (5) connected at its right-hand end to the waste channel (10).

In an alternative format of the present invention as shown in Figure 2, the micro-channel elements (6) are modified to permit the use of the 15 apparatus with cells growing on the surface of microcarrier beads (17). Thus, each micro-channel element (6) comprises a sample inlet channel (8) connected at its left-hand end to the sample reservoir (9), a cell growth chamber (7) connecting through a channel (16) to an assay chamber (11), and an outlet channel (12) leading to the common waste channel (10).

20

With reference to Figure 3, in a further embodiment of the apparatus, the cell growth chamber (2) may be provided with raised moulded structures disposed on the base portion of the cell growth chamber to form pillars (15), such that they form a barrier to the flow or passage of cells 25 arriving in the cell growth chamber (2) through the inlet channel (1), while allowing the passage of liquid. These structures are of dimensions chosen to provide gaps between the structures which are too narrow to allow passage of cells carried as a suspension in liquid moving in the device, but which are sufficiently large to allow cells growing on the surface of the cell 30 growth chamber (14) to migrate through the barrier by extension of cell processes between the barrier and subsequent cytokinesis. Suitable

dimensions for the gaps between the raised pillars (15) formed in the cell growth chambers are between 5 and 50  $\mu\text{m}$ , depending upon the cell type and cell size selected for capture.

5 Preferably, each micro-channel element (6) shown in Figure 3 additionally includes one or more assay chambers for performing assays involving cellular constituents and being connected in line between said cell growth chamber (2) and said outlet channel (5).

10 Suitably the disc (18) is of a one- or two-piece moulded construction and is formed of an optionally transparent plastic or polymeric material by means of separate mouldings which are assembled together to provide a closed structure with openings at defined positions to allow loading of the device with liquids and removal of waste liquids. In the simplest form, the

15 device is produced as two complementary parts, one or each carrying moulded structures which, when affixed together, form a series of interconnected micro-channel elements within the body of a solid disc. Alternatively the micro-channel elements may be formed by micro-machining methods in which the micro-channels and chambers forming the

20 micro-channel elements are micro-machined into the surface of a disc, and a cover plate, for example a plastic film, is adhered to the surface so as to enclose the channels and chambers.

25 To provide cultured cells with means for obtaining oxygen for metabolism and to permit use of  $\text{CO}_2$ -buffered media, one or more components of the device may be constructed from a gas permeable plastic, film, polymer, or membrane. Suitably, the gas permeable plastic, film, polymer, or membrane is formed from silicone polymers, such as polydimethylsiloxane, or from polyurethane, polytetrafluoroethylene or other

30 gas permeable plastic materials. In addition, means (not shown) are preferably provided for sealing the openings in the closed structure to

prevent evaporation of liquid during use, whereby such means seal the openings without interfering with gas exchange to the cell growth medium. Sealing can be accomplished by use of a further layer of gas permeable material across the whole device, or by use of a non-permeable material 5 applied locally to the openings only, leaving the remainder of the gas permeable material exposed to the local atmosphere.

Suitable plastic or polymeric materials for forming the cell growth chamber and micro-channels are preferably selected to have hydrophobic 10 properties, where the surface of the plastic or polymer can be additionally selectively modified by chemical or physical means to alter the surface properties to confer a desired property, for example, compatibility with cell growth, cell attachment and the attachment of biomolecules by covalent or non-covalent means. Preferred plastics are selected from polystyrene and 15 polycarbonate.

Alternatively, the cell growth chamber and micro-channels may be constructed from plastic or polymeric materials which are selected to have hydrophilic properties. The surface of the plastic or polymer can be 20 additionally selectively modified by chemical or physical means to alter the surface properties so as to produce localised regions of hydrophobicity within the chambers and/or microchannels to confer a desired property. By this means, for example, hydrophobic barriers or valves may be provided to control liquid flow within the apparatus. Preferred plastics are selected from 25 polymers with a charged surface, suitably chemically or ion-plasma treated polystyrene, polycarbonate or other rigid transparent polymers.

The micro-channel elements (6) are dispersed radially around the disc (18) and connected to a common centre port. The channels 1, 4, 5, 8, 12, 30 and 16 are of a dimension compatible with movement of cells along the channels. Suitably, the channels and chambers may be of any cross-

sectional shape, such as square, rectangular, circular, trapezoid and triangular. Suitably, the cell growth chamber (2) is sized to give a floor area between  $100\mu\text{m}^2$  and  $1,000,000\mu\text{m}^2$ , preferably between  $1000\mu\text{m}^2$  and  $1,000,000\mu\text{m}^2$  and most preferably between  $10,000\mu\text{m}^2$  and 5  $1,000,000\mu\text{m}^2$ . The plastic or polymer surface of the cell growth chamber may be selectively treated or modified to permit cell attachment and/or growth. Treatment preferably involves exposure to intense UV light to modify the polymer surface or alternatively the use of high voltage plasma discharge using known techniques (see Amstein, C.F. and Hartmann, P.A., 10 *J.Clin.Microbiol.*, 2, 1, 46-54 (1975)) to create a negatively charged surface suitable for cell growth and attachment and (if required) for assay purposes. Cell attachment may be further improved by the application of additional coatings to the surface of the cell growth chamber, eg. polylysine, collagen, fibronectin.

15

As already mentioned, in the preferred aspect of the invention, each of the micro-channel elements (6) is further provided with an assay chamber (3) which is located in line between the cell growth chamber (2) and the outlet channel (5), for performing assays involving cellular constituents. The assay chamber is sized proportionally to the volume of the cell growth chamber (2) to allow collection and/or capture of soluble analytes which may be derived from the cultured cells under study. Suitably, the volume of the assay chamber (3) is between twice and one tenth of the volume of the cell growth chamber (2). Advantageously, the 20 channel (4) which connects the cell growth chamber and the assay chamber is characterised by having hydrophobic walls and a cross-sectional area which is smaller than the channel (1) upstream of the cell growth chamber. Suitably, channel (4) has a cross-sectional area of between 0.99 and 0.01 times that of the inlet channel (1). Suitably, the outlet channel (5) 25 is characterised by having hydrophobic walls and a cross-sectional area of between 0.99 and 0.01 times that of channel (4). In this way, liquid flow 30

in the micro-channel elements can be controlled by application of a defined centrifugal force to cause the liquid to flow in a channel of a defined cross-sectional area, wherein the same force is insufficient to cause the liquid to flow in further linked channels of lesser cross-sectional area, this having the 5 effect of stopping the liquid flow at a desired position in the micro-channel element.

In an alternative means of controlling liquid flow, channel (1) and channel (4) are constructed with the same cross-sectional area, but the 10 surface of each of the channels may be selectively modified by chemical or physical means to confer a different degree of hydrophobicity on that channel. For example, channel (4) downstream of the cell growth chamber (2) may be treated to have a higher hydrophobicity than the channel (1) upstream of the cell growth chamber. By this means, application of a 15 defined force to liquid in channel (1), sufficient to cause liquid to move down this channel will be insufficient to cause that liquid to enter the second channel (4) of higher hydrophobicity, this having the effect of stopping the liquid flow at a desired position in the micro-channel element. Suitable means for selectively modifying the hydrophobicity of the surface 20 of the channels include exposure of defined areas to ionizing or electromagnetic radiation or ion plasma through a mask, or by application of a hydrophobic material or ink by screen printing or other means of localized application.

25 The means of controlling liquid flow within the apparatus as described above may be used alone or in combination as required, in order to confer the desired control of liquid flow in connected chambers and channels within the micro-channel element. If used in combination, the methods may be used sequentially, for example where a change in cross- 30 sectional area is followed by a change in hydrophobicity, forming two sequential points of liquid flow control. Alternatively, the methods may be

used coincidentally, in which a change in cross-sectional area of a channel is accompanied by a change in hydrophobicity of that channel, the combination being used to confer a degree of control over liquid flow not attainable using a single means.

5

The inner surface of the assay chamber may be coated with one or more ligands capable of specifically binding an analyte of interest by covalently or non-covalently linking the ligand to the surface. Examples of ligands suitable for the purpose include: biotin, streptavidin, protein A, 10 antibodies, lectins, hormone receptors, nucleic acid probes, DNA binding proteins and the like.

The apparatus and method can be used for the growth of cells and the detection and measurement of cellular activity, cellular parameters and 15 biochemical processes, for example cellular metabolism, cell viability, reporter gene expression, using non-invasive techniques, that is techniques which do not compromise the integrity or viability of cells. Alternatively, the apparatus may be used in the detection and measurement of cell-derived products, which have been released or otherwise excreted from 20 cells, such as peptide hormones, second messengers, etc. By use of the apparatus shown in Figure 3, the apparatus permits studies of cell migration in response to chemical or physical stimuli, for example chemotaxis, wherein a chemoattractant substance is placed on one side of a barrier and 25 cells placed on the opposite side of the barrier are observed to penetrate the barrier in moving toward the chemoattractant.

The invention may be used with any cell type that can be cultured on standard tissue culture plastic-ware. Such cell types include all normal and transformed cells derived from any recognised source with respect to 30 species (eg. human, rodent, simian), tissue source (eg. brain, liver, lung, heart, kidney skin, muscle) and cell type (eg. epithelial, endothelial). In

addition, cells which have been transfected with recombinant genes may also be cultured using the invention. There are established protocols available for the culture of diverse cell types. (See for example, Freshney, R.I., Culture of Animal Cells: A Manual of Basic Technique, 2<sup>nd</sup> Edition, Alan 5 R.Liss Inc. 1987). Such protocols may require the use of specialised coatings and selective media to enable cell growth and the expression of specialist cellular functions. None of such protocols is precluded from use with the apparatus of this invention.

10 The scale of the device will to a certain extent be dictated by its use, that is the device will be of a size which is compatible with use with eukaryotic cells. This will impose a lower limit on any channel designed to allow movement of cells and will determine the size of cell containment or growth areas according to the number of cells present in each assay. An 15 average mammalian cell growing as an adherent culture has an area of ~ 300 $\mu\text{m}^2$ ; non-adherent cells and non-attached adherent cells have a spherical diameter of ~10 $\mu\text{m}$ . Consequently channels for movement of cells within the device are likely to have dimensions of the order of 20-30 $\mu\text{m}$  or greater. Sizes of cell holding areas will depend on the number of 20 cells required to carry out an assay (the number being determined both by sensitivity and statistical requirements). It is envisaged that a typical assay would require a minimum of 500-1000 cells which for adherent cells would require structures of 150,000-300,000 $\mu\text{m}^2$ , i.e. circular 'wells' of ~400-600 $\mu\text{m}$  diameter.

25

The configuration of the micro-channels in the present invention is preferably chosen to allow simultaneous seeding of the cell growth chamber by application of a suspension of cells in a fluid medium to the sample reservoir by means of the sample inlet port, followed by rotation of the disc 30 (18) by suitable means at a speed sufficient to cause movement of the cell suspension outward towards the periphery of the disc by centrifugal force.

The movement of liquid distributes the cell suspension along each of the inlet micro-channels (1, 8) towards the cell growth chambers (2, 7). The rotation speed of the disc is chosen to provide sufficient centrifugal force to allow liquid to flow to fill the cell growth chamber (2, 7), but with

5 insufficient force for liquid to enter the restricted channel (4, 16) of smaller diameter on the opposing side of the cell growth chamber.

Once rotation has stopped, cells in the suspension can settle under gravity onto the bottom of the cell growth chamber (2, 7) and attach to the

10 treated surface if present. Cells which remain in the channel are unable to attach to the untreated hydrophobic surface and will remain in suspension. Therefore, following an appropriate period of time, chosen to allow cell attachment to take place, the device may be rotated at a higher speed than previously, such that all liquid and unattached cells is caused to move

15 towards the periphery of the disc into the waste channel (10) disposed around the edge of disc. Following clearance of channels of unattached cells, rotation is slowed to the same speed initially used to fill the micro-channel elements and fresh cell culture media is applied to the sample reservoir. By rotation of the disc once more, the cell culture media flows

20 under centrifugal force to fill the micro-channels and the cell growth chambers as previously described. This action leaves the attached cells seeded on the surface of the cell growth chambers covered in fresh culture media suitable for cell growth and for cell based assays.

25 In the apparatus of Figure 2, the beads (17) provide a large surface area for cell attachment and growth in a relatively small volume of liquid. In the device described herein, the beads (17) perform two functions; firstly they provide a surface for the attachment and growth of cells, so removing the need to provide a cell-growth compatible surface within the disc

30 allowing a wider range of materials to be used for construction, and secondly they provide a larger physical carrier for cells which allows

channels within the device to be easily designed to prevent access of cells. In the preferred embodiment, each element comprises a cell growth chamber (2, 7), an analysis chamber (3, 11), and a waste chamber (10) which are arranged radially about the centre of the disc and connected to a 5 common port in the centre of the disc.

Following the addition of cell samples to the apparatus, their attachment and subsequent cell growth, reagents and samples for assay may be introduced into the sample reservoir (9) through the sample inlet 10 port. Reagents to be supplied to all micro-channel elements may be introduced as described hereinbefore for seeding with cells, that is by applying a solution containing reagents to the central port and rotating the disc so as to cause distribution of the reagent to all micro-channels, cell growth chambers and assay chambers. The application of specific samples 15 to specific wells may be achieved by pipetting small volumes of liquid as discrete drops into the central well directly adjacent to the opening of the inlet channel leading to the cell growth chamber which is to receive the sample. This may be achieved, for example, by the use of a piezo-electric dispensing device (not shown), whereby dispensing of droplets of liquid 20 from the device is synchronised with the speed of rotation of the disc (18) such that, at an appropriate combination of dispensing drop frequency and rotation speed of the disc, individual droplets of liquid can be caused to impinge on the surface of the disc and can be caused to be transported by centrifugal force into an adjacent inlet channel (1, 8) and to mix with liquid 25 present in the cell growth chamber (2, 7).

Alternatively liquids may be pipetted as discrete drops onto the hydrophobic surface of a stationary disc (18), rotation of the disc being used to move these drops of liquid into the appropriate inlet channel and so 30 to the cell growth chamber. By these means, all reagents and samples may

be provided to the cell chamber in the desired order and proportions to perform the assay.

Once the liquid manipulations of the assay have been performed it is  
5 necessary to perform a detection procedure to measure the result of the assay, typically by measuring the signal emitted by a fluorescent, chemiluminescent or other labelling moiety. The device as shown in Figure 1b is configured to allow two means of detection of the assay signal. Firstly, the assay signal may be measured *in-situ* in the cell growth  
10 chamber. Such a signal would be typified by the measurement of the product of a reporter gene within the cells, e.g. fluorescence from GFP.

Alternatively, it may be desirable to measure cell-derived products or analytes, for example by immunochemical or other specific binding assay  
15 methods, in the absence of cells cultured in the apparatus, so as to avoid interference in measurement. In this case, the apparatus is provided with a second chamber, ie. the assay chamber (3, 11) disposed closer to the periphery of the disc and connected to the cell growth chamber (2, 7). The assay chamber is connected to the peripheral waste channel (10) by a  
20 narrow channel (5, 12), this channel having a smaller diameter than that (4, 16) connecting the assay chamber to the cell growth chamber. The differential in diameters between chambers allows, under controlled conditions of rotation and centrifugal force, as discussed above, liquid to be moved from the cell growth chamber to the assay chamber. For example,  
25 by this process, an analyte is moved from the cell growth chamber into the assay chamber where the analyte is subject to affinity capture by a ligand attached to the wall of the assay chamber. This process therefore provides a means of moving a cell-derived analyte, which has been released or otherwise excreted from cells, away from the cellular environment,  
30 immobilising the analyte within the assay chamber by means of a specific

binding partner for binding to the analyte, and allowing subsequent additions of reagents and processing to permit the detection of the analyte.

Optionally, at least one of the reagents for use in an assay method

5 utilising the apparatus may be labelled with a detectable label by covalent or non-covalent attachment. Suitable detectable labels may be selected from fluorescent labels, chemi-luminescent labels, bio-luminescent labels, enzyme labels and radioactive labels. Suitable fluorescent labels for tagging reagents according to the method of the invention may be selected from the

10 general categories of fluorescent dyes, including, but not limited to, fluoresceins, rhodamines, cyanine dyes, coumarins, and the BODIPY groups of fluorescent dyes. Examples of bioluminescent detectable labels are to be found in the fluorescent reporter proteins, such a Green Fluorescent Protein (GFP) and aequorin. Alternative labels for providing a detectable signal can

15 be fluorescence energy transfer labels.

Once the assay has been completed, measurement of the signal may be achieved by means appropriate to the labelling molecule or moiety used in the assay and is typically by optical means. For example, luminescence

20 emitted from cells or from a fluorescent labelled assay reagent may be detected in the microfabricated apparatus by the use of imaging apparatus incorporating a CCD camera, or by the use of a fluorimeter. Detection of emitted fluorescence may be achieved through the body of the disc (18) where the disc is wholly constructed of a transparent material or

25 alternatively through windows of transparent material which have been fabricated into the body of the disc.

As an alternative to non-radioactive detection, the apparatus of the present invention may be used in conjunction with radioactive detection

30 utilising the scintillation proximity technique. For example, scintillant-containing beads may be introduced into the apparatus. Alternatively the

microfabricated apparatus of the invention may have incorporated a scintillant containing layer into or onto an interior surface of the cell growth chamber (2, 7) and/or the assay chamber (3, 11). The region of the apparatus containing the scintillant beads or scintillant surface is preferably

5 optically transparent so as to enable the material to transmit light at a given wavelength with optimum efficiency. The scintillant-containing region can be composed of any transparent material containing scintillant, eg. a scintillant glass based on lanthanide metal-containing compounds, or a plastic material such as polystyrene or polyvinyltoluene, into which is

10 incorporated the scintillant substance.

Suitable scintillant substances can include aromatic hydrocarbons such as p-terphenyl, p-quaterphenyl and their derivatives, and derivatives of oxazoles and 1,3,4-oxadiazoles, for example, 2-(4-t-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole and 2,5-diphenyloxazole. A wavelength shifter such as 1,4-bis(5-phenyl-2-oxazolyl)benzene, or 9,10-diphenylanthracene, may also be included.

A binding moiety such as one member of a specific binding pair may

20 be immobilised onto the surface of the bead or scintillant layer so as to specifically bind with an analyte which may be derived from cells used in the assay process. Suitable specific binding pair members with which the invention is useful include biotin, streptavidin, protein A, antibodies, lectins, hormone receptors, nucleic acid probes, DNA binding proteins and the like.

25 It is to be understood that either member of the specific binding pair may be attached and immobilised for binding to a complementary member of the specific binding pair.

Typical radioisotopes that may be used to label the assay reagent

30 include those commonly used in biochemistry such as [<sup>3</sup>H], [<sup>125</sup>I], [<sup>35</sup>S] and [<sup>33</sup>P] but does not preclude the use of other isotopes. Detection

methodologies based on scintillation proximity are well known (see for example US Patent No.4568649, Bertoglio-Matte,J.). Detection may utilise a number of modalities to be combined to allow all assays to be measured simultaneously or in rapid succession. Alternatively an appropriate imaging 5 format may provide suitable detection means for both radioactive and non-radioactive assays utilising the apparatus.

In some uses, some assay areas will be redundant; that is, not all elements of the apparatus will be used every time. It is envisaged that the 10 user will decide what type of assays it is required to perform and then select appropriate control means for directing liquid flow within the apparatus. Consequently, such structures will be compatible with a range of assay procedures and protocols which require different complexities of fluid movement. For example, detection of a GFP linked reporter gene will 15 require a simple well structure to allow measurement of fluorescence. In contrast, measurement of an analyte secreted from cells, for example the measurement of a cytokine by immunoassay, or measurement of cellular mRNA following lysis, will require a more complicated structure to separate the cells from the secreted analyte prior to the analysis of that analyte

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The invention is further illustrated by reference to the following examples.

#### Example 1

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A stock culture of HeLa cells was grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% calf serum and L-glutamine in a plastic flask using standard tissue culture conditions. Cells were harvested by trypsinisation and the resulting suspension concentrated 30 by centrifugation to give a cell concentration of  $10^7$  cells/ml. Aliquots of the cell suspension (1 $\mu$ l) were applied to openings in the surface of a

microfabricated structure of the type shown in Figure 1, produced by injection moulding, with internal channels of depth 50 $\mu$ m and width 100 $\mu$ m.

The cells suspension was moved along the inlet channels within the 5 disc to circular cell growth chambers of depth 50 $\mu$ m and diameter 500 $\mu$ m and the cells allowed to attach and grow. Following incubation for 48hours in a tissue culture incubator (37°C/95% RH) the cells were examined for cell density, morphology and viability. Visible examinations by phase contrast microscopy showed cell populations growing in microstructures to 10 have the same density and morphology as control cultures grown from the same parent stock and maintained in standard tissue culture plasticware. Cells were subsequently tested for viability using a commercial test kit (LIVE/DEAD Viability Kit, Molecular Probes, Oregon, L-3224) by flushing 15 growth medium from the cells within the device, washing the cells with a phosphate buffered saline solution (PBS) and introducing a solution of the fluorescent assay reagents into the cell growth chambers. Subsequent examination by fluorescent microscopy showed that cells growing in microstructures had maintained a cell viability of >95%, comparable with control cells grown under standard tissue culture conditions.

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#### Example 2

A microfabricated plastic disc of the type shown in Figure 1, produced by injection moulding which contained a number of radial internal 25 channels of depth 50 $\mu$ m and width 100 $\mu$ m was selectively surface modified by exposure to a 500W UV lamp at a distance of 20cm for 30minutes through a metal mask such that UV light impinged on the surface only on regions defined by the mask.

30 A stock culture of HeLa cells was grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% calf serum and L-

glutamine in a plastic flask using standard tissue culture conditions. Cells were harvested by trypsinisation and the resulting suspension concentrated by centrifugation to give a cell concentration of  $10^7$  cells/ml. Aliquots of the cell suspension (1 $\mu$ l) were applied to openings in the surface of the

5 device and cells moved along the channels by rotation about the disc axis (1000 rpm for 30 seconds). Following incubation for 18 hours in a tissue culture incubator (37°C/95% RH) cells were examined by phase contrast microscopy. Cells were found to be preferentially growing in regions of the disc previously exposed to UV light, cells in other regions were observed to

10 be sparser and less well attached. The disc was then rotated at 1000rpm for 30 seconds and cells re-examined. Following spinning cells in UV exposed areas were observed to have remained attached to the plastic surface and remained morphologically identical to control cells; in contrast cells on non-exposed surfaces were totally removed by the centrifugal force

15 generated by the spinning disc.

### Example 3

HeLa cells grown and harvested as described above were introduced

20 into the device of Example 2 into micro channels which contained moulded pillars of various dimensions dispersed in the channels so as to act as barriers to cell movement in the channels. A suspension of cell was introduced into the channels and the structure incubated for 18 hours to allow the cells to settle and grow. Subsequent examination revealed that in

25 channels where the gaps between pillars were 10 x 60 $\mu$ m or greater cells were observed to have moved freely past the barriers. In contrast where gaps between pillars were 10 x 20 $\mu$ m or less, cells were prevented from passing the barrier when transported by liquid flow in suspension. However on subsequent incubation and growth cells were observed to migrate past

30 such barriers by slow deformation. Such barrier structures may prove useful

for study of cell movement or migration in response to chemical or physical stimuli.

Example 4

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HeLa cells transfected to yield stable expression of a Calreticulin-GFP fusion protein were introduced into a moulded apparatus of the type shown in Figure 1, supporting channels of 100µm width and 100µm depth connected to circular chambers of 600µm and 1000µm diameter and 10 100µm depth. Cells were incubated for 18hours in a tissue culture incubator and examined by confocal fluorescence microscopy. Cells grown in microfabricated structures showed the same level of GFP expression as control cells grown under conventional tissue culture conditions.

15 Example 5

HeLa cells were grown and introduced into a microfabricated disc as described in Example 1. Following incubation for 18 hours growth medium was removed from cells and replaced with media containing different 20 concentrations of the membrane permeabilising agent digitonin in the range 0-0.2mg/ml (w/v) and cells incubated in the presence of digitonin for 10 minutes. Thereafter the viability of the cells was measured as described in Example 1. Results were found to be equivalent to cells exposed to the same dose range of digitonin in a microtitre plate based assay.

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Claims

1. Apparatus microfabricated for performing cell growth and cell based assays in a liquid medium, said apparatus comprising:
  - 5 a) a base plate supporting a plurality of micro-channel elements, each comprising a cell growth chamber, an inlet channel for supplying liquid sample thereto and an outlet channel for removal of liquid sample therefrom;
  - b) a cover plate positioned over said base plate said cover plate
  - 10 extending over said elements so as to define said chambers and connecting channels; said cover plate being supplied with holes to provide access to said channels; and
  - c) means, incorporated in said cell growth chambers, for cell attachment and cell growth.
- 15 2. Apparatus according to claim 1 wherein said base plate comprises a rotatable disc which is microfabricated to provide a sample introduction port located towards the centre of the disc and connected to an annular sample reservoir, and wherein said micro-channel elements are radially dispersed on said disc with their respective input channels connected to receive sample from said reservoir.
3. Apparatus according to claim 1 or claim 2 wherein said cover plate is fabricated from a gas permeable plastics material.
- 25 4. Apparatus according to any one of claims 1 to 3 wherein said means for cell attachment and cell growth comprises selectively treating or modifying at least a portion of a surface of said cell growth chamber.
- 30 5. Apparatus according to any one of claims 1 to 3 wherein means for cell attachment and cell growth comprises one or more microcarrier beads

located in said cell growth chamber, wherein each of said microcarrier beads provides for cell attachment and cell growth.

6. Apparatus according to any one of claims 1 to 5 wherein the cell  
5 growth chamber further includes raised moulded features disposed on the base portion of the cell growth chamber to form pillars.

7. Apparatus according to any one of claims 1 to 6 wherein the cross-  
10 sectional area of said inlet channel is greater than that of said outlet channel.

8. Apparatus according to claim 7 wherein the cross-sectional area of said outlet channel is between 0.99 and 0.01 times that of said inlet channel.

15 9. Apparatus according to any one of claims 1 to 8 wherein at least some of said micro-channel elements each further comprises one or more assay chambers for performing assays involving cellular constituents and connected in line between said cell growth chamber and said outlet  
20 channel.

10. Apparatus according to claim 9 wherein the assay chamber or chambers are connected to each other and to said cell growth chamber by an intermediate channel or channels in the order of: inlet channel, cell  
25 growth chamber, intermediate channel 1, assay chamber 1, intermediate channel 2 (if present), assay chamber 2 (if present)..... , outlet channel, and wherein the cross-sectional areas of the respective channels reduce progressively from the inlet channel to the outlet channel.

11. Apparatus according to claim 10 wherein the cross-sectional area of the or each intermediate channel and the outlet channel is between 0.99 and 0.01 times that of the immediately preceding (upstream) channel.

5 12. Apparatus according to any one of claims 1 to 11 wherein there is provided in or on an interior surface of one or more of said chambers a layer comprising a scintillant substance.

10 13. Apparatus according to claim 12 wherein the layer comprising a scintillant substance includes a binding moiety bound thereto, said binding moiety being a member of a specific binding pair selected from biotin, streptavidin, protein A, antibodies, lectins, hormone-receptors, nucleic acid probes, and DNA-binding proteins.

15 14. A method for determining the effect of a test substance on a cellular activity or physical parameter by the use of an apparatus as defined in claims 1-13, which method comprises:

- a) providing a suspension of cells in a fluid medium;
- b) introducing said cells into said apparatus and causing said cells to be transported to one or more cell growth chambers in said apparatus;
- c) providing one or more samples of test substances whose effect upon the cells is to be measured under conditions so as to cause said cells to be exposed to said substances;
- d) determining the effect of the test substances on said cells by means of optical detection.

20 25 15. A method according to claim 14 wherein cells are cultured adhering to a surface within the apparatus prior to the introduction of the test substances.

16. A method according to claims 14 or 15 wherein there are provided following step c) one or more assay reagents and dispersing said reagents to one or more reaction chambers in said apparatus.

5 17. A method according to claim 16 wherein at least one of said assay reagents is labelled with a detectable label selected from fluorescent labels, chemiluminescent labels, bioluminescent labels, enzyme labels and radioactive labels.

10 18. A method for measuring a cellular analyte by the use of the apparatus as defined in claims 1-13, which method comprises:

- a) providing a suspension of cells containing an analyte to be measured in a fluid medium;
- b) introducing said cells into said apparatus and causing the cells to be transported to one or more cell growth chambers in the apparatus;
- c) allowing cells to grow;
- d) providing one or more assay reagents and dispersing said reagents to one or more chambers in said apparatus;
- e) measuring the cellular analyte by optical means.

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20 19. A method according to claim 18 wherein at least one of said assay reagents is labelled with a detectable label selected from fluorescent labels, chemiluminescent labels, bioluminescent labels, enzyme labels and radioactive labels.

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Figure 1/3

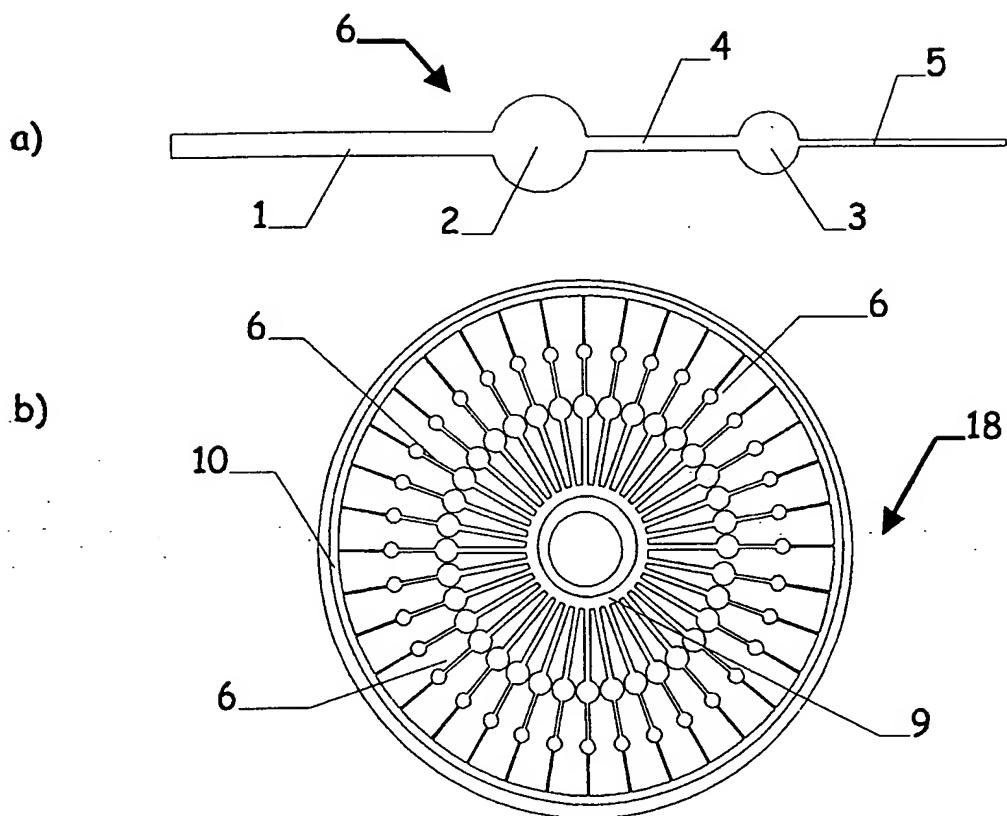
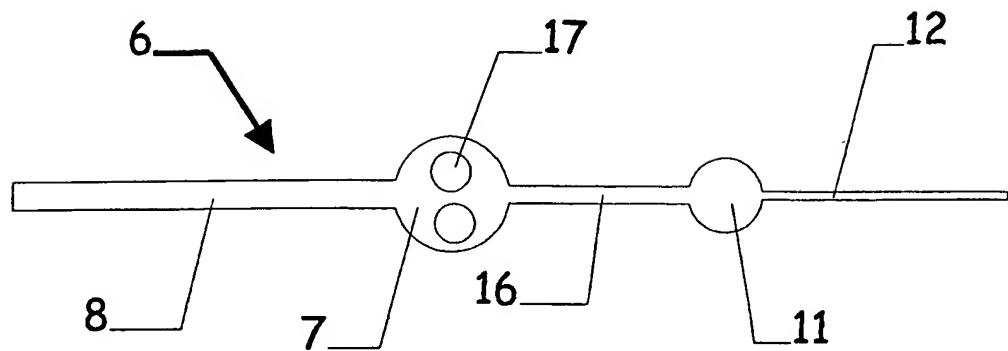
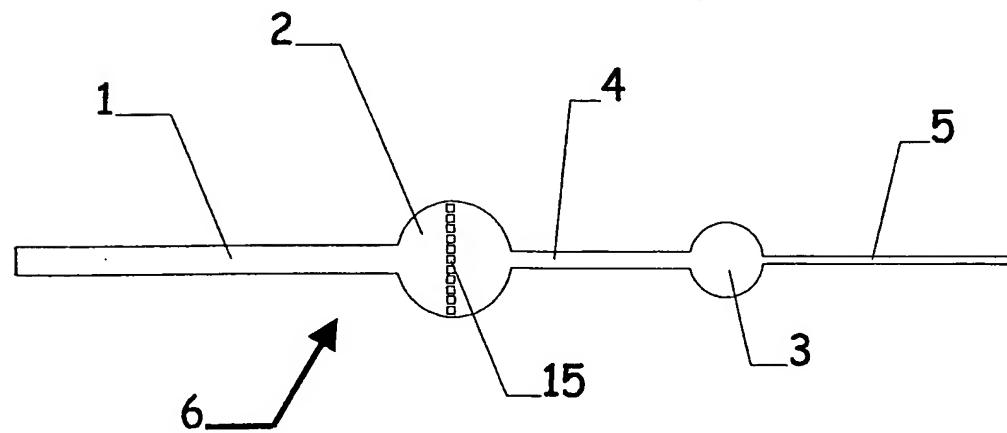


Figure 2/3Figure 3/3

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/00954

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 6 C12M1/20 C12M1/34 B01L3/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 B01L C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 657 879 A (IMEDEX) 9 August 1991 (1991-08-09)	1-3, 5, 14, 16, 18
Y	page 6, line 26 - page 10, line 5; claims; figures	1, 2, 4, 5, 9, 13-15, 17-19
X	US 5 637 469 A (WILDING PETER ET AL) 10 June 1997 (1997-06-10)	1, 4, 6, 9, 13-19
Y	claims; figures; example 5	2, 4, 5, 9, 13, 15, 17, 19
X	US 4 018 652 A (LANHAM JAMES W ET AL) 19 April 1977 (1977-04-19) column 9, line 56 - column 11, line 11; claims; figures 6, 8 column 8, line 25 - column 8, line 38	1, 14, 18
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		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the International search

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# INTERNATIONAL SEARCH REPORT

Intern	Application No
PCT/GB 99/00954	

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 077 845 A (JOHNSON LEIGHTON CLIFFORD) 7 March 1978 (1978-03-07) column 4, line 26 - column 5, line 46; claims; figures ---	1,14,18
A	EP 0 745 856 A (BIO MERIEUX VITEK INC) 4 December 1996 (1996-12-04) column 7, line 36 - column 7, line 48; claims; figures ---	1,14
A	EP 0 282 840 A (BECTON DICKINSON CO) 21 September 1988 (1988-09-21) claims; figures ---	1
A	DE 295 00 587 U (SCHREIBER HANS) 15 May 1996 (1996-05-15) claims; figures ---	
Y	US 4 318 994 A (MEYER MICHAEL C ET AL) 9 March 1982 (1982-03-09) claims; figures ---	1,14,18
Y	WO 96 14933 A (UNIV PENNSYLVANIA) 23 May 1996 (1996-05-23) claims; figures ---	1,14,18
X	US 5 593 838 A (ZANZUCCHI PETER J ET AL) 14 January 1997 (1997-01-14) claims; figures ---	1,9,12
Y	WO 97 21090 A (GAMERA BIOSCIENCE) 12 June 1997 (1997-06-12) claims; figures -----	1,14,18
Y	-----	1,2

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Int. Appl. No.

PCT/GB 99/00954

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
FR 2657879 A 09-08-1991			NONE		
US 5637469	A 10-06-1997	US 5866345 A		02-02-1999	
		AT 155711 T		15-08-1997	
		AT 167816 T		15-07-1998	
		AT 140025 T		15-07-1996	
		AT 140880 T		15-08-1996	
		AT 174813 T		15-01-1999	
		AU 677780 B		08-05-1997	
		AU 4222393 A		29-11-1993	
		AU 680195 B		24-07-1997	
		AU 4222593 A		29-11-1993	
		AU 677781 B		08-05-1997	
		AU 4222693 A		29-11-1993	
		AU 4222793 A		29-11-1993	
		AU 677197 B		17-04-1997	
		AU 4223593 A		29-11-1993	
		CA 2134474 A		11-11-1993	
		CA 2134475 A		11-11-1993	
		CA 2134476 A		11-11-1993	
		CA 2134477 A		11-11-1993	
		CA 2134478 A		11-11-1993	
		DE 69303483 D		08-08-1996	
		DE 69303483 T		06-02-1997	
		DE 69303898 D		05-09-1996	
		DE 69303898 T		20-02-1997	
		DE 69312483 D		04-09-1997	
		DE 69312483 T		12-02-1998	
		DE 69319427 D		06-08-1998	
		DE 69319427 T		10-12-1998	
		DE 69322774 D		04-02-1999	
		DE 69322774 T		17-06-1999	
		EP 0637996 A		15-02-1995	
		EP 0637997 A		15-02-1995	
		EP 0639223 A		22-02-1995	
		EP 0637998 A		15-02-1995	
		EP 0637999 A		15-02-1995	
		ES 2106341 T		01-11-1997	
		ES 2127276 T		16-04-1999	
		GR 3025037 T		30-01-1998	
		HK 16897 A		13-02-1997	
		JP 7506430 T		13-07-1995	
		JP 7506431 T		13-07-1995	
		JP 7506256 T		13-07-1995	
		JP 7506257 T		13-07-1995	
		JP 7506258 T		13-07-1995	
		WO 9322053 A		11-11-1993	
		WO 9322054 A		11-11-1993	
		WO 9322421 A		11-11-1993	
		WO 9322055 A		11-11-1993	
		WO 9322058 A		11-11-1993	
US 4018652 A 19-04-1977			NONE		
US 4077845 A 07-03-1978			AU 504980 B		01-11-1979
			AU 3145577 A		21-06-1979
			CA 1090239 A		25-11-1980
			DE 2817145 A		26-10-1978

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern	al Application No
PCT/GB 99/00954	

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US 4077845	A	FR 2388046 A		17-11-1978
		GB 1548530 A		18-07-1979
		IT 1103597 B		14-10-1985
		JP 1130914 C		17-01-1983
		JP 53144186 A		15-12-1978
		JP 57024749 B		26-05-1982
EP 0745856	A 04-12-1996	US 5609828 A		11-03-1997
		AU 695716 B		20-08-1998
		AU 5459196 A		02-01-1997
		AU 7190998 A		01-10-1998
		AU 7191098 A		01-10-1998
		AU 7191198 A		01-10-1998
		AU 7191298 A		01-10-1998
		AU 7191398 A		01-10-1998
		BR 9602272 A		07-04-1998
		CA 2176672 A		01-12-1996
		JP 9119928 A		06-05-1997
		US 5766553 A		16-06-1998
		US 5869005 A		09-02-1999
		US 5746980 A		05-05-1998
		US 5843380 A		01-12-1998
EP 0282840	A 21-09-1988	US 4806316 A		21-02-1989
		AT 89601 T		15-06-1993
		AU 604857 B		03-01-1991
		AU 1300588 A		15-09-1988
		CA 1308635 A		13-10-1992
		DE 3881085 A		24-06-1993
		DK 146888 A		18-09-1988
		FI 881257 A, B		18-09-1988
		JP 1742022 C		15-03-1993
		JP 4026902 B		08-05-1992
		JP 63258650 A		26-10-1988
DE 29500587	U 15-05-1996	NONE		
US 4318994	A 09-03-1982	NONE		
WO 9614933	A 23-05-1996	US 5726026 A		10-03-1998
		US 5744366 A		28-04-1998
		US 5587128 A		24-12-1996
		AU 704277 B		15-04-1999
		AU 4236996 A		06-06-1996
		AU 698213 B		29-10-1998
		AU 4282896 A		06-06-1996
		AU 4282996 A		06-06-1996
		CA 2181189 A		23-05-1996
		CA 2181190 A		23-05-1996
		CN 1157639 A		20-08-1997
		CN 1143917 A		26-02-1997
		EP 0739240 A		30-10-1996
		EP 0739423 A		30-10-1996
		JP 9511407 T		18-11-1997
		JP 9509498 T		22-09-1997
		WO 9615269 A		23-05-1996
		WO 9614934 A		23-05-1996
		US 5928880 A		27-07-1999

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

Interr. Application No

PCT/GB 99/00954

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5593838	A 14-01-1997	US 5585069 A AU 705351 B AU 4152396 A AU 705659 B AU 4233796 A CA 2204912 A CA 2205066 A EP 0791238 A EP 0808456 A JP 11500602 T WO 9615450 A WO 9615576 A US 5681484 A US 5643738 A US 5846396 A US 5755942 A US 5863708 A US 5858804 A	17-12-1996 20-05-1999 06-06-1996 27-05-1999 06-06-1996 23-05-1996 23-05-1996 27-08-1997 26-11-1997 19-01-1999 23-05-1996 23-05-1996 28-10-1997 01-07-1997 08-12-1998 26-05-1998 26-01-1999 12-01-1999
WO 9721090	A 12-06-1997	AU 702403 B AU 1283397 A CA 2239613 A CN 1208464 A EP 0865606 A NO 982563 A AU 4144897 A EP 0917648 A WO 9807019 A	18-02-1999 27-06-1997 12-06-1997 17-02-1999 23-09-1998 05-08-1998 06-03-1998 26-05-1999 19-02-1998